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Study code: 3000-4201
(Old code 1903001)

Study title:

Effects on blood pressure and heart rate.
Hydroxymatairesinol.

CONFIDENTIAL

Study Report

EFFECTS ON BLOOD PRESSURE AND HEART RATE

HYDROXYMATAIRESINOL

Study number: **P11.2-1999**

Date: 20.8.2002 (version 2)

Sponsor:

Hormos Medical Ltd.
Tykistökatu 6A
FIN-20520 Turku
FINLAND

Sponsor Study number: 1903001

PreFa
Preclinical Pharmacology Research Unit
University of Turku

Key Words HMR, safety pharmacology, blood pressure, heart rate

1. GENERAL

1.1. SIGNATURE

Title Effects on blood pressure and heart rate; HMR

PreFa study number: P11.2-1999

Sponsor study number: 1903001

Test item: HMR

This Report version 2 replaces the 1st version dated 14.6.2000. Following change was made:

1. **Section 2.3.4. Rationale for dose selection:** Reference to a study demonstrating the antitumor activity of HMR has been added.

This report is a complete and accurate account of the methods employed and the data obtained

20.8.2002

Aapo Honkanen, Study Director Date

1.2. TABLE OF CONTENTS

1.3. PURPOSE OF THE STUDY

The purpose of this study was to assess the general and safety pharmacological properties of the compound HMR by assessing its effect on blood pressure (diastolic, systolic and MAP) and heart rate in anaesthetized rats.
In addition to HMR, the effects of another compound, (HTS-101) were tested in the same experiment. Same control group (vehicle treatment) and reference compound-treated group were used in the evaluation of the effects of these compounds. The results from HMR and HTS-101 are reported separately.

1.4. GUIDELINES

The study procedures described was based on the guidelines listed below:

- Asetus Kokeellisiin ja muihin tieteellisiin tarkoituksiin käytettävien selkärankaisten eläinten suojelemiseksi tehdyn eurooppalaisen yleissopimuksen voimaansaattamisesta. Suomen säädöskokoelma n:o 1360/90. Helsinki, 21 joulukuuta 1990.
- European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, European Treaty Series No. 123, (EU n:o 609/86) (Official Journal of the European Communities No L 358) Strasbourg 24th November 1986.

1.5. PERMISSION FROM THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

The study has a permission from the animal care and use committee of University of Turku n:o 922/99.

1.6. SPONSOR

Hormos Medical Ltd.
Tykistökatu 6A
FIN-20520 Turku
FINLAND

1.7. TEST LABORATORIES

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1.8. STUDY DIRECTOR

Aapo Honkanen, M.Sc. (Pharm.)

1.9. PRINCIPLE SCIENTIST

Kristiina Raatesalmi, Researcher

1.10. PERSONNEL INVOLVED IN THE STUDY

PreFa/Department of Pharmacology and Clinical Pharmacology
Esa Korpi, Professor of Pharmacology
Aapo Honkanen, Study Director
Kristiina Raatesalmi, Researcher, Principal Scientist

CRST/Biostatistics
Esa Wallius

1.11. TIME TABLE

Experimental starting date:	6.10.1999
Experimental completion date:	28.10.1999

1.12. SUMMARY

18 male Sprague-Dawley rats weighing 295 - 406 g were used. The animals were anaesthetized with isoflurane and their blood pressure and heart rate were measured from the femoral artery with a Micron MP transducer connected to a Grass amplifier 7P122. Data was collected and analyzed with PC analysis software, Acknowledge. Animals were given vehicle, polyethylene glycol 300 (PEG 300), reference compound tamoxifen or HMR (all i. v. in PEG 300, 1 ml/kg) with the doses of 3, 10 or 30 mg/kg, at 30-min intervals. Mean arterial blood pressure (MAP) and heart rate (HR) were calculated at 10 and 30 min after each drug administration.
Five rats treated with tamoxifen died immediately after the dose of 30 mg/kg so two separate statistical analyses were conducted. In the first analysis, all dose levels were included, but only vehicle- and HMR-groups were analyzed, while in the second

analysis, all treatment groups were analyzed, but only with 2 dose levels, i.e. 3 and 10 mg/kg.
The first analysis for MAP did not show any treatment effect ($p = 0.57$), but there was a significant treatment x time interaction ($F = 4.23$, $p = 0.03$), which was probably due to a small increase in MAP after the high dose of HMR as compared to effect of vehicle. The first analysis for HR did not show any treatment effect
When the effects of 3 and 10 mg/kg doses were tested, there was no significant treatment effect for MAP indicating that at this dose level neither tamoxifen nor HMR altered MAP. When the effects of 3 and 10 mg/kg doses on HR were tested, there was no significant treatment effect for HR indicating that at these dose levels neither tamoxifen nor HTS-101 altered HR. The results indicate that hydroxymatairesinol (HMR) does not affect mean arterial pressure or heart rate at the doses up to 30 mg/kg (i.v.).

2. MATERIALS AND METHODS

2.1. TEST SYSTEM/SUBJECTS

Experimental animals:	Rats, Sprague-Dawley Hsd:SD
Age/weight:	10-14 weeks/295-414 g
Source:	Harlan Winkelman GmbH, Germany
Number of animals in the study:	18
Number of animals/group:	6
Acclimatization period:	At least one week before the start of experiments.
Principles for selection into test groups:	Animals were allotted randomly into different treatment groups.
Grounds for selection of species:	Rats are commonly used in studies of this type

2.2. ENVIRONMENTAL CONDITIONS

Animal care:	The animals were cared and checked daily by the experimenters and/or personnel of the Central Animal Laboratory. The bedding of the animals was changed twice and water bottles once a week.
Number of animals/cage:	3 rats/cage.
Cage Type:	Polycarbonate Macrolon III (Scanbur AS, Denmark).

Bedding:	Aspen chips (Tapvei Oy Kaavi, Finland). The results of the analysis for specified contaminants are attached (Appendix 3).
Water:	Community tap water, *ad libitum*, except during the experiments. The results of the analysis for specified contaminants are attached (Appendix 4.).
Fodder:	RM1 (E) SQC, Special Diet Service, Witham Essex, England. Certificate detailing nutritional composition and levels of specified contaminants is attached (Appendix 5.).
Ambient temperature:	21 ± 2.5 °C
Humidity:	50 % ± 15 %
Illumination:	12-h dark/light cycle; lights on from 7.00 to 19.00 and lights of from 19.00 to 7.00.
Room numbers:	Experimental room: 314 Colony room: 309

2.3. REAGENTS

2.3.1. Test compounds

Hydroxymatairesinol (HMR) (mw. 374)

Vehicle:	PEG 300 Sigma (Chemicals Co, St Louis, MO, USA)
Batch:	00799
Storage:	at 4 °C, desiccated, protected from direct light

2.3.2. Reference compound

Tamoxifen citrate (mw. 563.65,)

Manufacturer:	Hormos Medical Ltd.
Vehicle:	PEG 300 Sigma (Chemicals Co, St Louis, MO, USA)
Batch:	1022IU
Storage:	at room temperature protected from direct light

2.3.3. Other reagents

Isoflurane (Forene)

Manufacturer:	Abbott
Lot:	50134VA
Receiving date:	07/99
Storage:	at room temperature protected from direct light

2.3.4. Rationale for dose selection

In the experiments assessing the pharmacodynamic efficacy of HMR ,e.g. antitumor activity (Saarinen et al. Nutrition and cancer 2000 (36):207-216) a dose 15 mg/kg, (p.o.) have been found to be effective.

In the present study, treatments were given i.v. Therefore, the doses of 3, 10 and 30 mg/kg (i.v.) were expected to produce at least similar or higher systemic drug concentrations than those in the indication model experiments.

2.3.5. Preparation and handling of test compound solutions

Fresh test compound solutions were prepared on each experimental day. HMR and reference compound tamoxifen were dissolved in polyethylene glycol (PEG) 300. If necessary, solutions were sonicated at 40 °C for 8-15 min.

2.4. EXPERIMENT

2.4.1. Procedure

Anaesthesia

During the experiment, rats were anaesthetized with isoflurane/oxygene mixture (O_2 0.8 l/min, isoflurane 2 %) using a Penlon Sigma vaporizer (Penlon Ltd, UK). The rats were first placed in a pre-anaesthesia chamber (isoflurane concentration 4.5 %) until the loss of righting reflex (within about 3 min). Thereafter the rat was transferred onto operation table and connected to an anaesthesia mask.

Measurement of blood pressure and heart rate

The body temperature of the rats was regulated by a Letica temperature control unit HB 101/2 (Letica Sientific Instruments, Spain) and the temperature was adjusted to be 37.5 °C. Left femoral artery and vein were prepared. The femoral vein was cannulated with a 15-cm long PE50 cannula (Clay Adams, USA) filled by saline-heparin solution (30 IU/ml). Following the operation the rat was allowed to stabilize for 20 min. Blood pressure and heart rate were recorded with a Micron MP 15 transducer (Micron Instruments, CA, USA) connected to a Grass amplifier 7P122 (Grass Model 7D Polygraph, Grass Instrument co, Quincy, Mass., USA). Blood pressure waveform was printed on an ink-paper and collected with theAcqKnowledge PC-software (Biopac MP100 system, Biopac, USA), which was used to digitize outputs from the Grass amplifier. Afterwards the data was analyzed and transferred to MS-Excel worksheet.

2.4.2. Administration of compounds

Intravenously, in volume of 1 ml/kg.

HMR and tamoxifen infusions (intravenously, in volume of 1 ml/kg) were given cumulatively at 30-min intervals. Control animals received repeated vehicle infusions at same intervals. The cannula was flushed after each administration with 0.15 ml 0.9 % NaCl.

Treatments

	Cumulative dosing		
Groups	**Dose 1**	**Dose 2**	**Dose 3**
I vehicle	-	-	-
II Tamoxifen	3 mg/kg	10 mg/kg	30 mg/kg
III HMR	3 mg/kg	10 mg/kg	30 mg/kg

$n_i = 6$, $n = 18$

2.4.3. Data collection

The blood pressure waveform was collected and MAP calculated with the AcqKnowledge analysis software (MP 100 system, Biopac Inc., USA). Numerical data was then transferred to MS-Excel software, which was used in further processing of the data. The baseline MAP and HR were measured from each animal during 15-second periods just before the first treatment. Treatment-induced changes in MAP (mm Hg) and HR (beats per min, bpm) were recorded and calculated 10 and 30 min after each dose. If the animal did not react even to the highest dose of test compounds, the animal was given a phenylephrine at the dose of 6 μg/kg and if this dose did not alter MAP, the animal was rejected from the data set, since this indicated that the test compound unlikely reached systemic circulation.

2.4.4. Statistics

The blood pressure data were tested with one-way analysis of variance for repeated measures (ANOVA) treatment group (vehicle, HMR, tamoxifen) being a between factor and time (6 measurements performed during cumulative drug administration) being a within factor. The analyses were carried out with SAS software (SAS Institute Inc., version 6.12, Gary, NC, USA).

2.4.5. Termination of the experiments

Following completion of the experiments, the animals were killed by cervical dislocation.

3. ARCHIVING

Study plan, final report and original data from different experiments are retained in the archive of PreFa (Tykistökatu 6B) at least for 10 years following approval of final report.

After that, the further treatment of the documentation is decided together with the Sponsor. The documentation or parts of it may be delivered to the Sponsor on request before 10-year term. No data or documentation will be destroyed without written permission from the Sponsor.

4. DEVIATIONS FROM THE STUDY PLAN

The experiment was performed as described in the Study Plan.

5. RESULTS

Average weights of the animals in different treatment groups shown in table 3.1. did not differ significantly between the groups ($F = 0.78$, $p = 0.48$).

Table 3.1. Average weights of the animals in the different treatment groups.

	Mean	SD	SEM	Min	Max	n_i
Vehicle	356	39	16	295	398	6
Tamoxifen	380	23	9	346	406	6
HMR	366	36	15	316	399	6

Baseline values for MAP and the effects of vehicle and drug treatments on MAP are shown in the table 3.2. Baseline blood pressure did not differ between the groups ($F= 0.47$, $p = 0.63$). Five rats treated with tamoxifen died immediately after the dose of 30 mg/kg, and therefore, two separate statistical analyses were conducted. In the first analysis, all dose levels were included, but only vehicle- and HMR-groups were analyzed, while in the second analysis, all treatment groups were analyzed, but only at 2 dose levels, i.e. 3 and 10 mg/kg.

The first analysis did not show any treatment ($F = 0.35$, $p = 0.57$) or time effects ($F = 2.6$, $p = 0.11$), but there was a significant treatment x time interaction ($F = 4.23$, $p = 0.03$). Interaction was probably due to a small increase in MAP after high dose of HMR as compared to effect of vehicle.

When the effects of 3 and 10 mg/kg doses were tested, there was no significant treatment effect for MAP ($F = 0.21$, $p = 0.81$) indicating that with this dose level neither tamoxifen nor HMR altered MAP. However, there was a significant time effect ($F = 7.77$, $p < 0.001$) but no treatment x time interaction ($F = 1.45$, $p = 0.22$). The time effect was due to slight increase of MAP during the experiment, especially in vehicle- and tamoxifen-treated groups.

Table 3.2. Mean arterial pressure (MAP) after repeated vehicle treatment or after ascending doses of HMR or reference compound tamoxifen. The data is expressed as changes in mmHg relative to baseline measured just before the first treatment.

MAP		**3 mg/kg**		**10 mg/kg**		**30 mg/kg**	
	Baseline	**10 min**	**30 min**	**10 min**	**30 min**	**10 min**	**30 min**
Vehicle							
Mean	92	7	9	15	12	11	4
S.D.	8	8	8	9	10	10	13
S.E.M	3	3	3	4	4	4	5
MIN	83	-3	-6	-2	-1	-2	-16
MAX	100	21	16	26	25	24	18
n_i	6	6	6	6	6	6	6
Tamoxifen							
Mean	89	7	2	14	12	-9	-3
S.D.	4	3	12	2	6	-	-
S.E.M	2	1	5	1	3	-	-
MIN	83	1	-21	11	2	-	-
MAX	94	10	10	18	19	-	-
n_i	6	6	6	6	6	1	1
HMR							
Mean	91	11	5	12	8	17	17
S.D.	5	4	8	5	8	4	5
S.E.M	2	1	3	2	3	2	2
MIN	82	7	-9	3	-2	12	8
MAX	95	16	12	18	16	23	21
n_i	6	6	6	6	6	6	6

Baseline values for HR and the effects of vehicle and drug treatments on HR are shown in the table 3.3. Baseline blood pressure did not differ significantly between the groups ($F= 3.41$, $p = 0.06$). The first analysis for HR did not show any treatment ($F = 0.56$, $p = 0.47$) or time effects ($F = 2.2$, $p = 0.15$), or interaction between treatment and time ($F = 0.84$, $p = 0.42$).

When the effects of 3 and 10 mg/kg doses on HR were tested, there was no significant treatment effect ($F = 0.36$, $p = 0.70$), indicating that at these dose levels neither tamoxifen nor HTS-101 altered HR. Similarly there was no time effect ($F = 2.88$, $p < 0.08$) or treatment x time interaction ($F = 1.48$, $p = 0.24$).

Table 3.3. Heart rate (HR) after repeated vehicle treatment or after treatment with ascending doses of HMR or reference compound tamoxifen. The data is expressed as changes in beats per min relative to baseline measured just before the first treatment.

		3 mg/kg		10 mg/kg		30 mg/kg	
	Baseline	**10 min**	**30 min**	**10 min**	**30 min**	**10 min**	**30 min**
Vehicle							
Mean	379	-20	-20	-30	-24	-37	-28
S.D.	28	11	14	11	20	17	16
S.E.M	12	4	6	5	8	7	6
MIN	333	-28	-46	-40	-53	-63	-46
MAX	416	2	-4	-14	6	-9	-2
n_i	6	6	6	6	6	6	6
Tamoxifen							
Mean	348	-8	-24	-35	-26	-117	-87
S.D.	15	5	9	13	10	-	-
S.E.M	6	2	4	5	4	-	-
MIN	332	-14	-35	-50	-37	-	-
MAX	371	-2	-14	-17	-14	-	-
n_i	6	6	6	6	6	1	1
HMR							
Mean	366	-27	-8	-30	-14	-40	-4
S.D.	36	25	16	14	34	17	58
S.E.M	15	10	7	6	14	7	24
MIN	316	-71	-32	-49	-58	-61	-59
MAX	399	-4	17	-7	48	-22	102
n_i	6	6	6	6	6	6	6

6. DISCUSSION AND CONCLUSIONS

The results indicate that hydroxymatairesinol (HMR) does not affect mean arterial pressure or heart rate at the doses up to 30 mg/kg (i.v.). Lethal effects of 30 mg/kg dose of reference compound tamoxifen suggest that this dose is already associated with toxicity in rats. Tamoxifen is poorly soluble in water. Therefore, the reason of immediate death of the animals after the infusion of high concentration of this compound may also be clotting of the tamoxifen in the veins.

7. DISTRIBUTION OF THE REPORT

The Report is written in duplicate, one original copy being retained in the Archives of PreFa and one delivered to the Sponsor.

Appendices

1. Values from the individual animals
2. Statistics
3. Report from analysis of bedding for contaminants
4. Report from analysis of water for contaminants
5. Report from analysis of fodder for nutritional composition and levels of specified contaminants.